

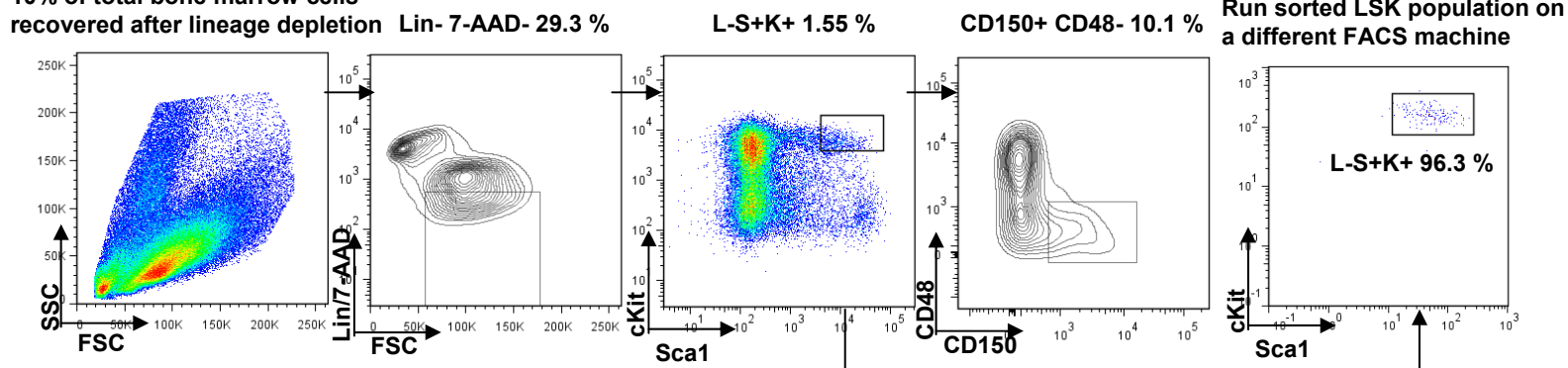
SUPPLEMENTAL INFORMATION

Supplemental Figure 1

A

10% of total bone marrow cells

recovered after lineage depletion



B

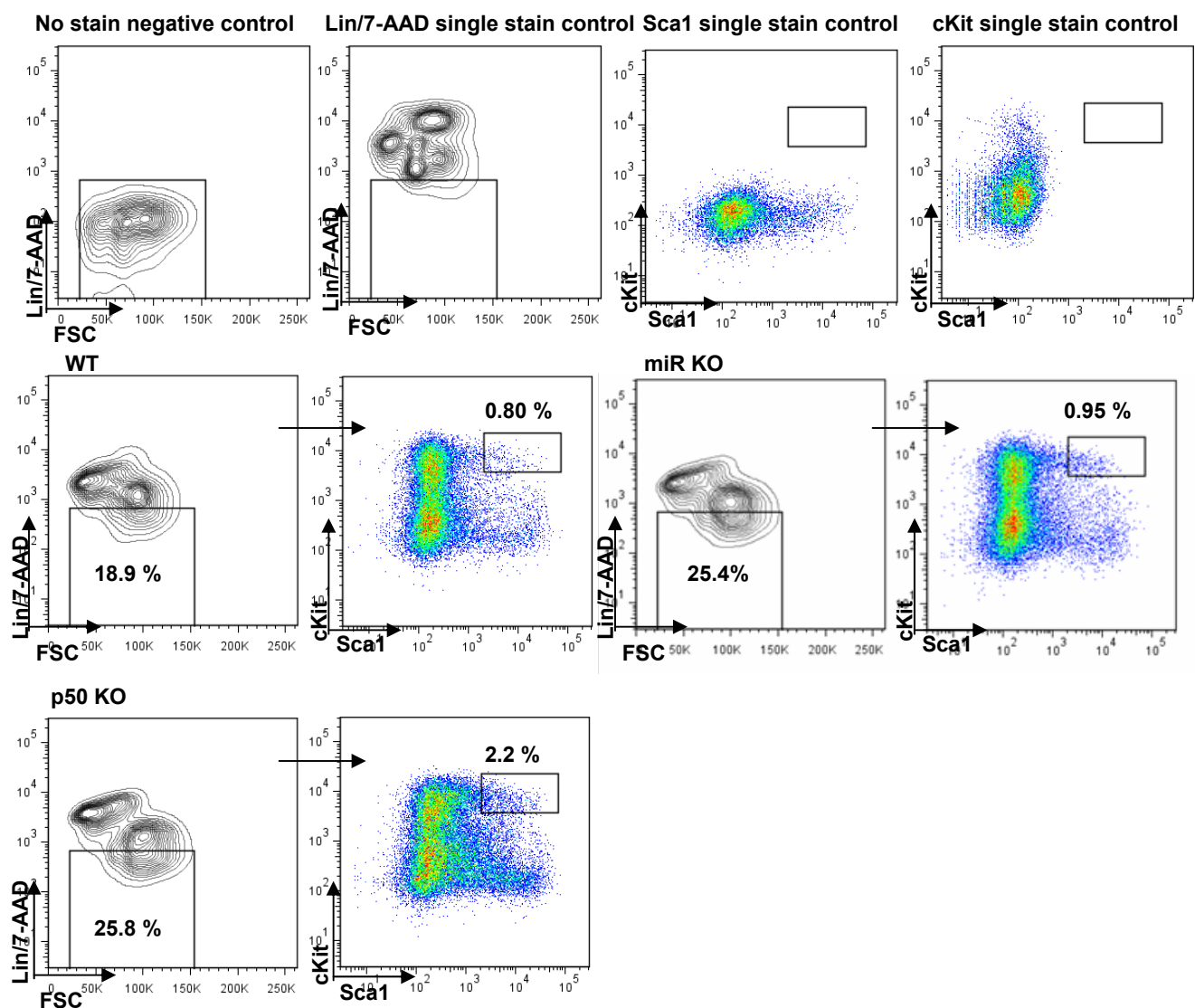


Figure S1. Representative FACS sorting plots (related to Figure 1 and 4). A. Representative FACS plots for sorting WT LSK (defined as Lineage-cKit+Sca1+) and HSC (defined as LSK CD150+CD48-). Before FACS sorting, total bone marrow nucleated cells (after red blood cell lysis) were magnetically depleted of lineage positive cells with a cocktail of lineage antibodies (CD11b, Gr1, CD19, B220, CD4, CD8, NK1.1, Ter119, and IL7R α) using MACS (Miltenyi). In general, 10% of cells were recovered after MACS and were stained with fluorophore-conjugated antibodies for cell sorting on a BD FACSaria cell sorter. 7-AAD was included as viability dye to exclude dead cells. Sorted cells were re-analyzed on a separate FACS machine (MACSQuant10) to check for purity before loading onto a microfluidic single cell cytokine chip. B. Representative FACS plots for sorting WT, p50 KO and miR-146a KO LSK cells.

Supplemental Figure 2

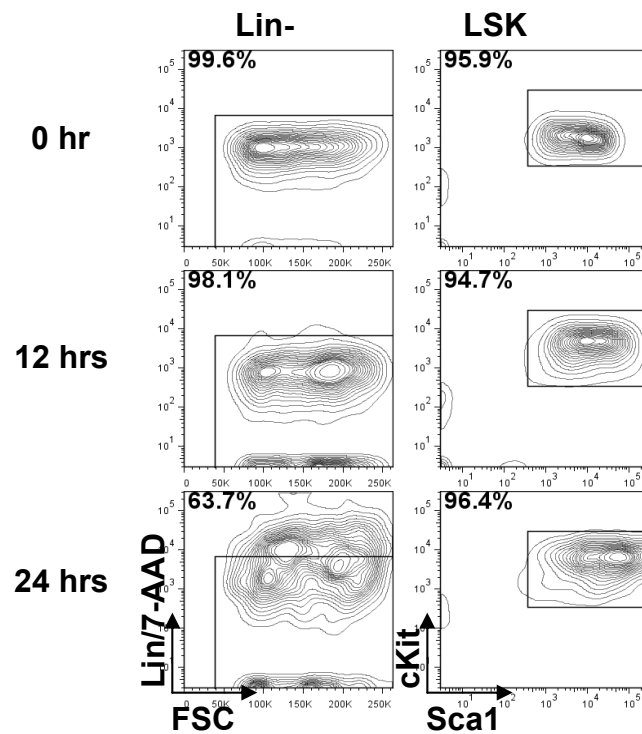


Figure S2. Representative FACS plots of WT LSK cells after stimulation (related to Figure 1). WT LSK cells were analyzed by FACS after stimulation with LPS and Pam3CSK4 *in vitro* for 0, 12, and 24 hours.

Supplemental Figure 3

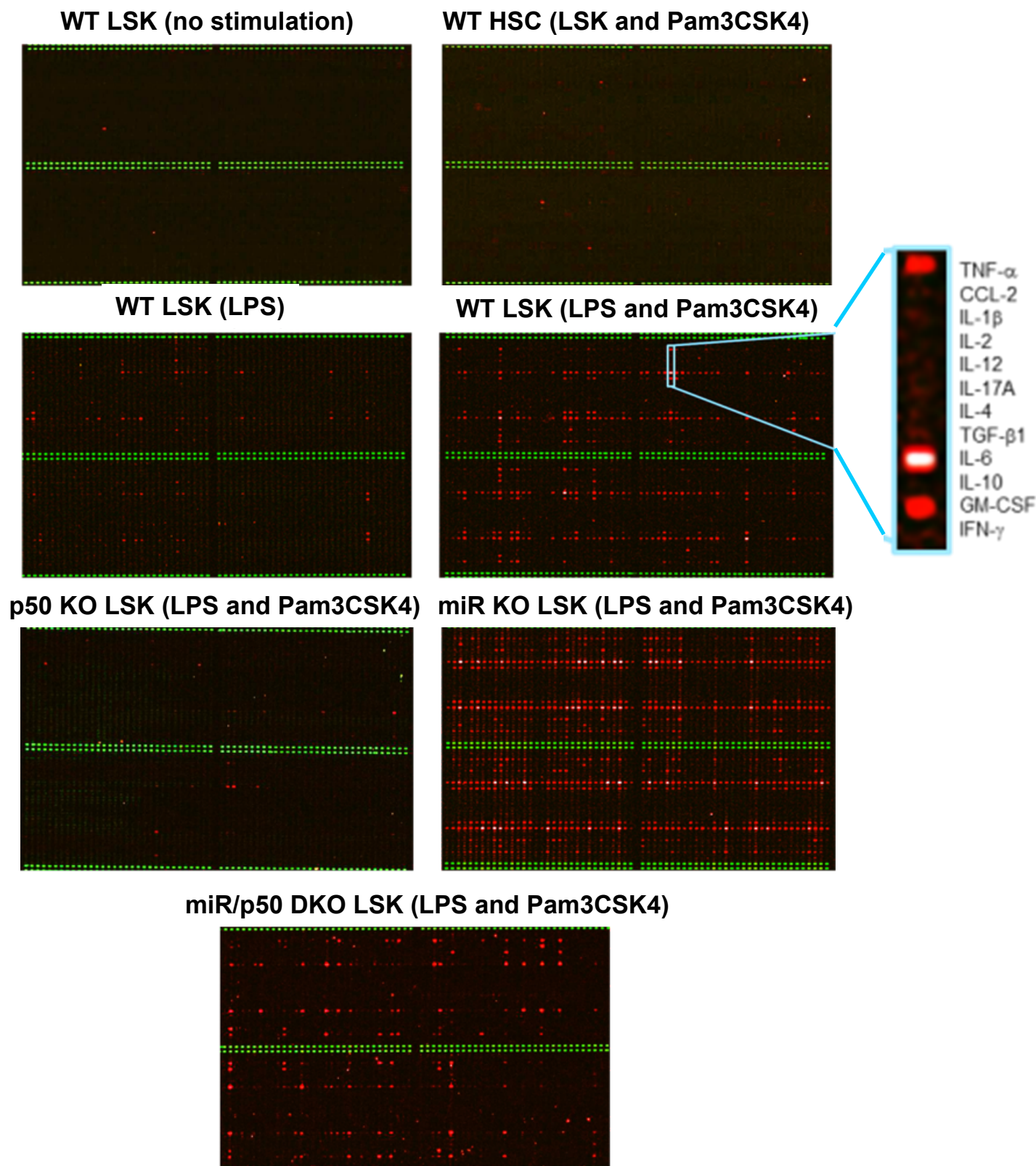
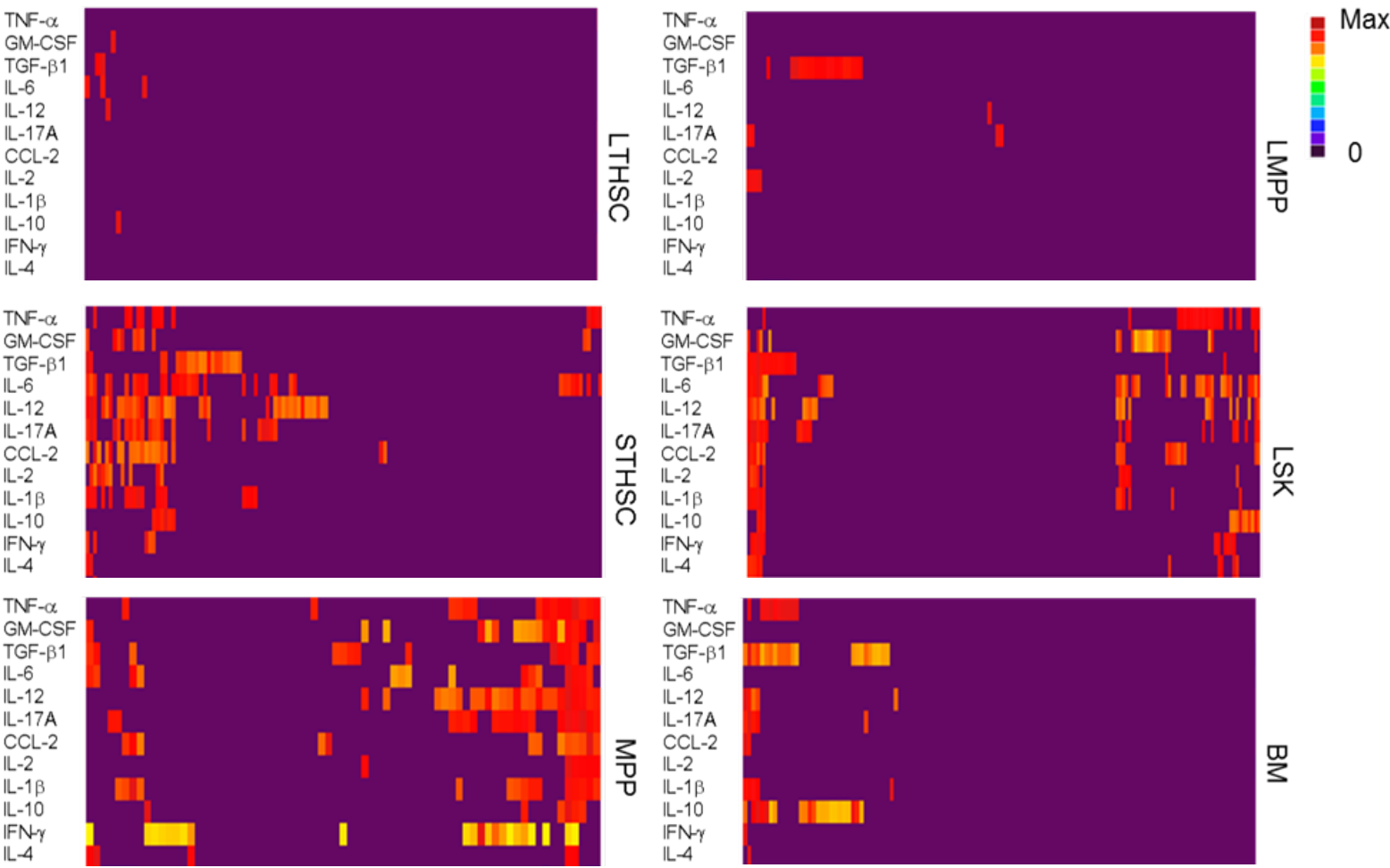


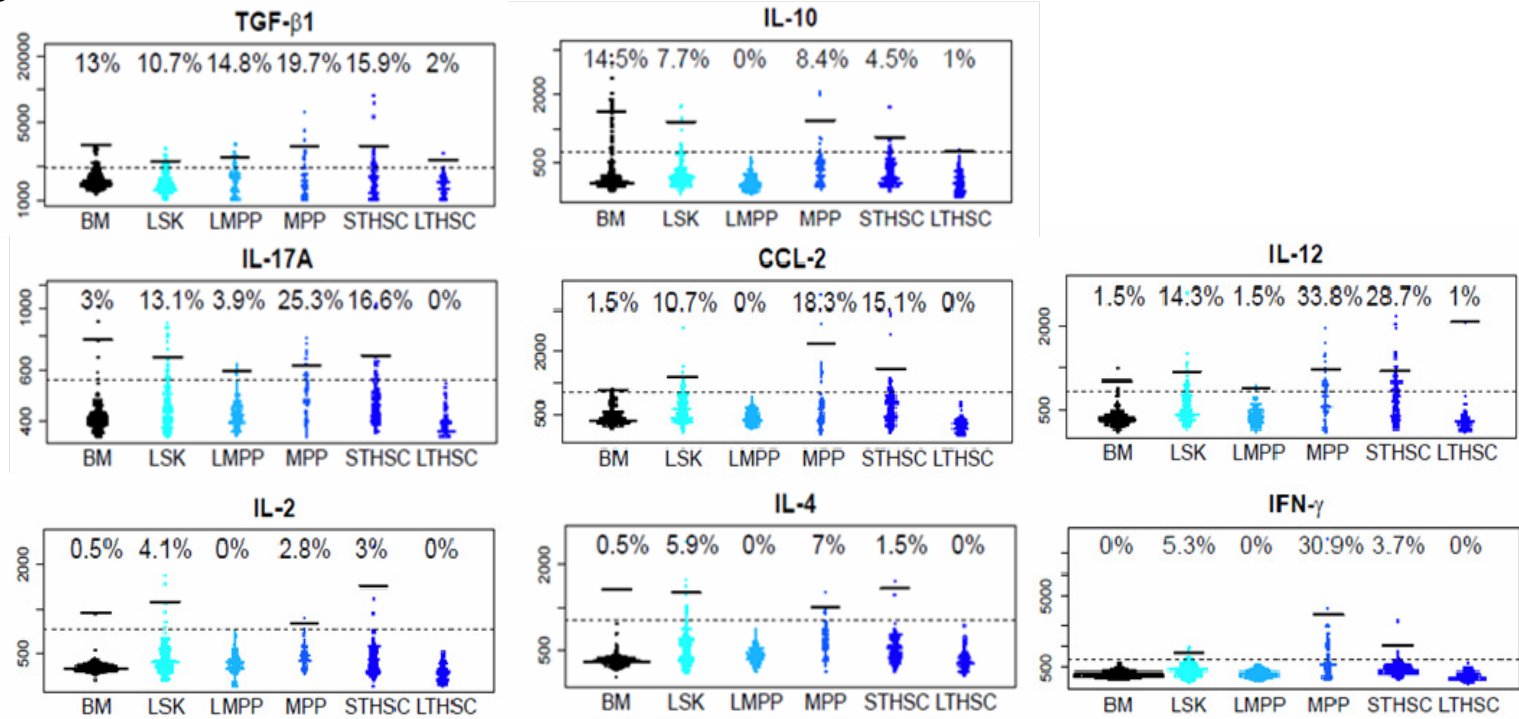
Figure S3. Representative fluorescence images of single cell cytokine chips of different cell types (related to Figure 1 and 4). Each image is labeled with the name of the cell type and stimulation condition on top. The image represents about 5% of the chip. The magnified picture of WT LSK (LPS and Pam3CSK4) represents one microchamber with ELISA detection spots for 12 different cytokines from top to bottom. Each chip contains >5000 identical microchambers. Black means no fluorescence detected; red fluorescence is the detected protein signal, with increasing in red fluorescence intensity correlating with increasing level of a given cytokine; green fluorescence represents reference and control spots and should be identical in intensity across all chambers to represent uniform development across the entire chip.

Supplemental Figure 4

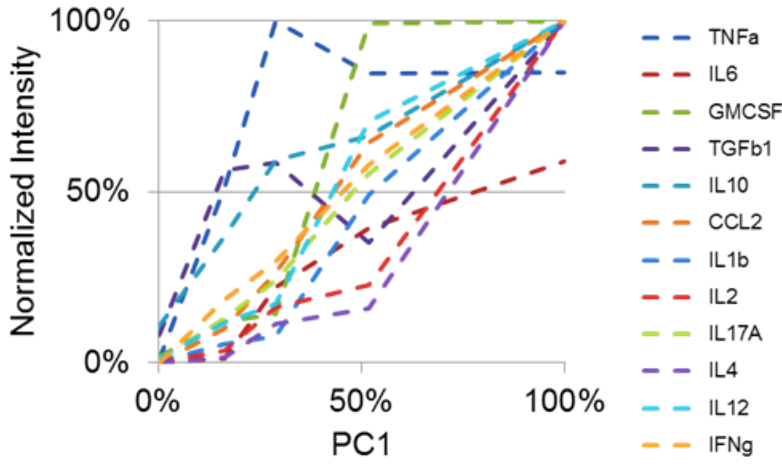
A



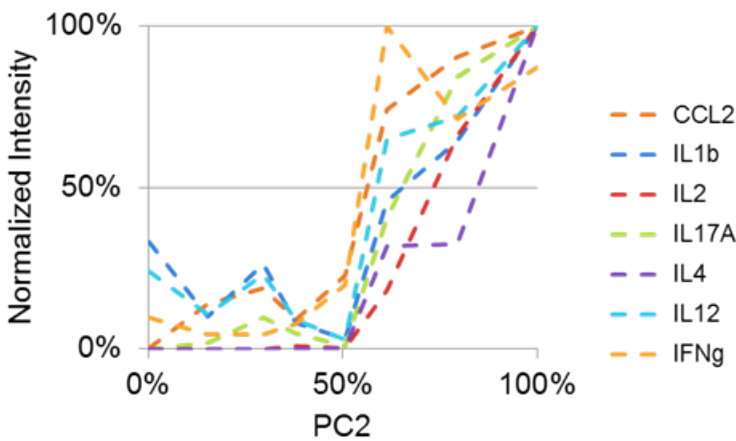
B



C



D



E

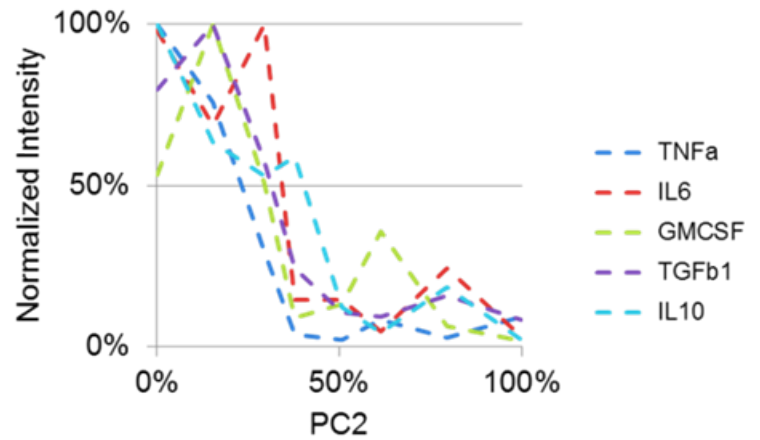


Figure S4. Single cell analysis of cytokine production by various HSPC subsets in response to TLR stimulation (related to Figure 2). A. global view of cytokine production by LT-HSCs, ST-HSCs, MPPs, LMPPs, LSK cells and total BM cells under LPS and Pam3CSK4 stimulation. The data are presented as heatmaps. Each row represents an individual cytokine and each column represents an individual cell. The color on the heatmap represents the amount of cytokine produced, from purple (undetectable), yellow (intermediate), to red (maximum). B. Comparison of HSPC subsets by individual cytokine. 8 out of 12 cytokines are shown here and the rest is shown in Figure 2B. Each plot is composed of several thousand individual dots that represent the secretion of a specific cytokine from several thousand single cells. The six cell groups arranged from left to right are Total BM, LSK cells, LMPPs, MPPs, ST-HSCs, LT-HSCs under LPS and Pam3CSK4 stimulation. The numbers on top show the percentage of cytokine producing cells identified by the gate (the dotted line) and the bars show the mean intensity of only the cytokine-producing cells (the cells above the dotted line). C-E. Principal component analysis of all six cell groups. C. Principal component 1 (PC1) represents the overall intensity of all 12 cytokines measured. The intensity of each cytokine (y-axis) increases as PC1 percent (x-axis) increases from 0 to 100%. D and E. Principal component 2 (PC2) represents the level of biased cytokine production profile. As PC2 percent (x-axis) increases from 0 to 100%, the lymphoid group of cytokines, including CCL2, IL-1 β , IL-2, IL-17a, IL-4, IL-12 and IFN- γ , increases in intensity (D) and the myeloid group of cytokines, including TNF- α , IL-6, GM-CSF, TGF- β 1 and IL-10, decreases in intensity (E).

Supplemental Figure 5

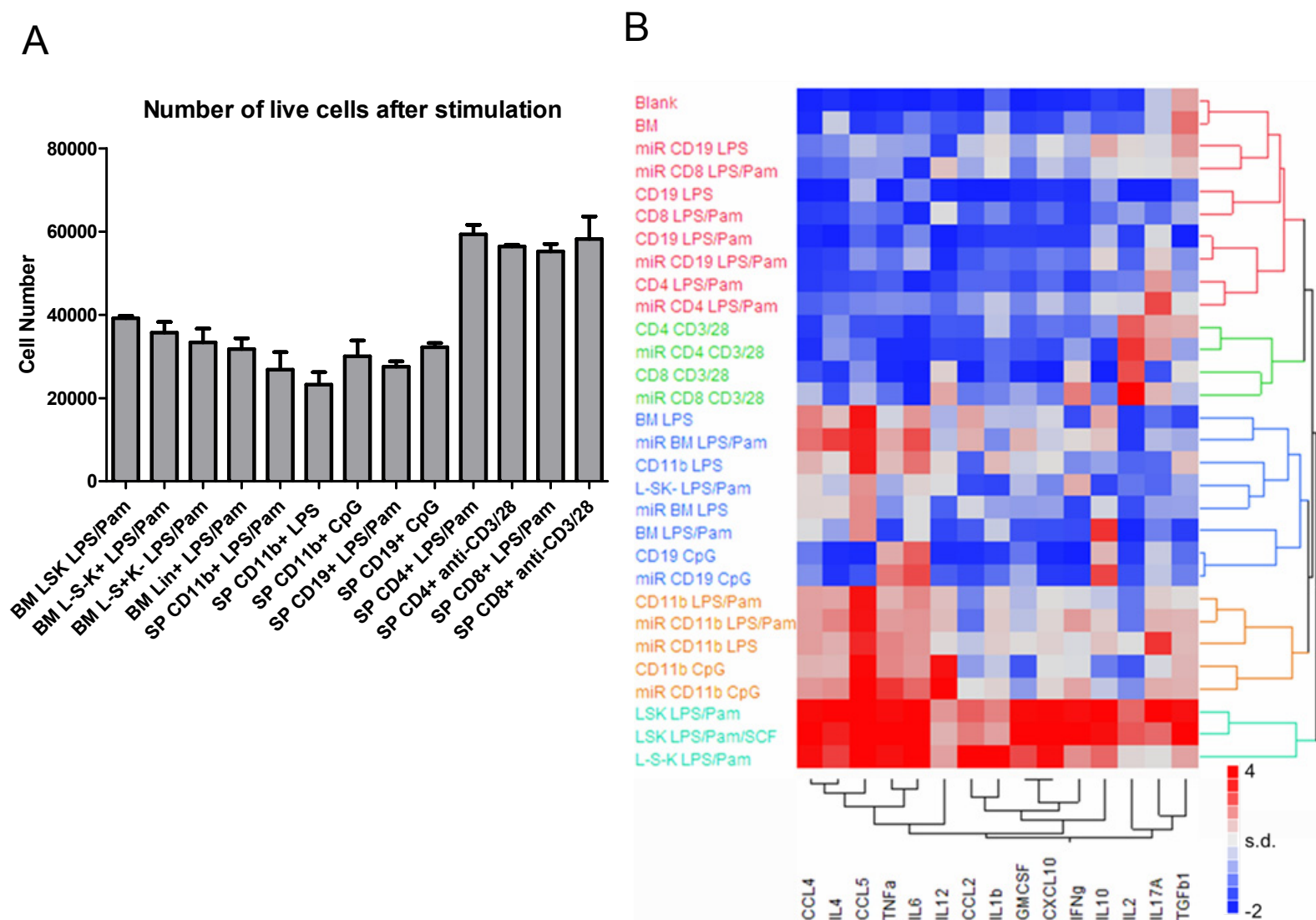


Figure S5. Functional significance of HSPC-produced cytokines (related to Figure 5). A. HSPCs and mature immune cells were cultured *in vitro* with various stimuli for 24 hours and number of live cells was analyzed by FACS. B. Multiplexed ELISA quantification of cytokines in cell culture medium. This graph includes both mature cells from WT and miR-146a KO mice. The data is analyzed by two-way hierarchical clustering that groups similar cell groups and proteins together. The grouping is shown by the tree structure for both cell groups and proteins. Each row represents an individual cell type under a specific stimulation and each column represents an individual protein measured. The result is presented by a heat map with color representing the amount of cytokine secretion, from blue (low) to white (intermediate) to red (high).

Supplemental Figure 6

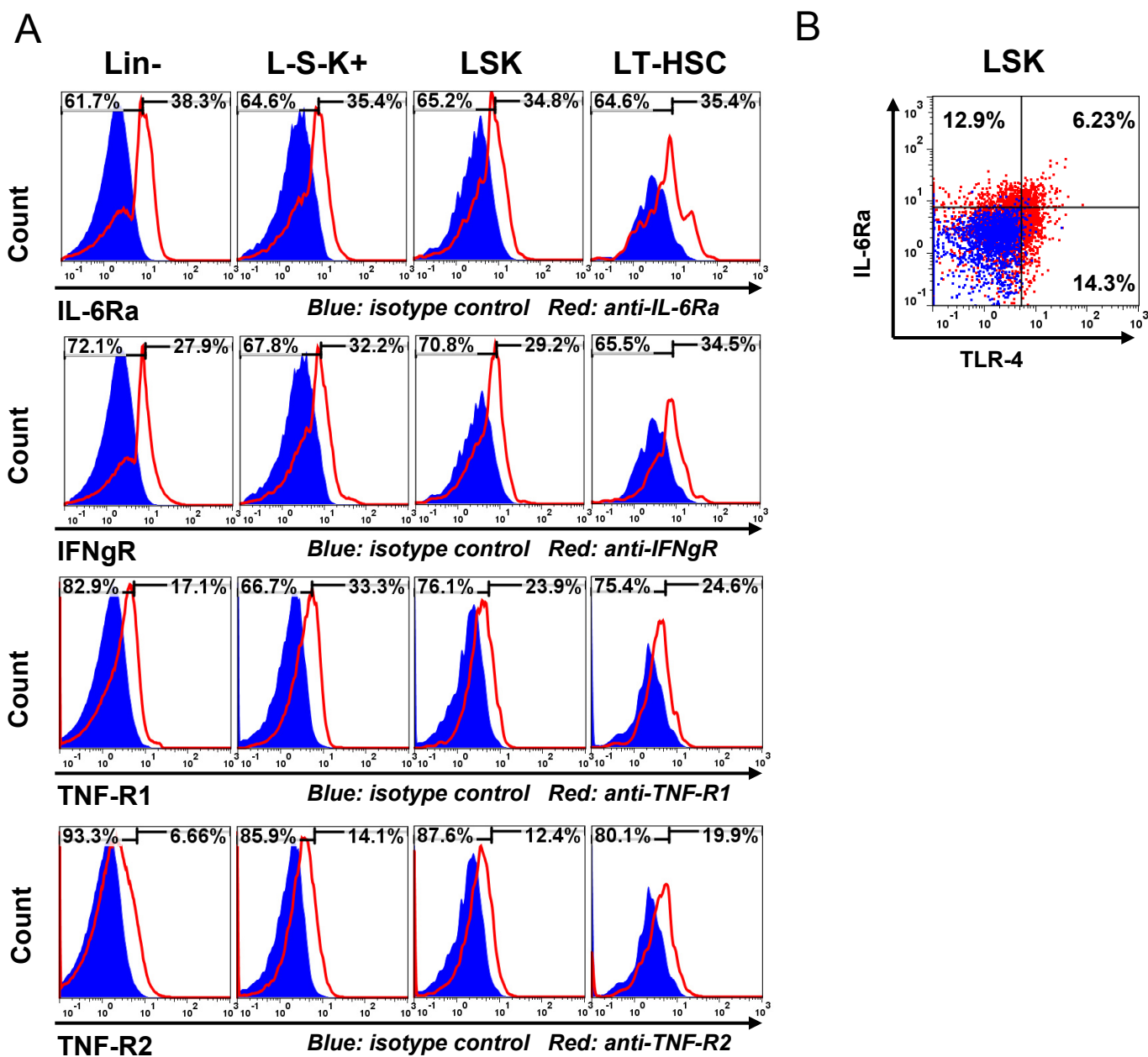


Figure S6. Basal expression of various cytokine receptors on the cell surface of HSPCs by FACS analysis (related to Figure 6). A. Representative FACS histograms showing cell surface expression of cytokine receptors IL-6R α , IFN- γ R, TNF-R1 and TNF-R2 on Lin-, L-S-K+, LSK and LT-HSC cell populations. B. Cell surface expression of TLR-4 and IL-6R α on LSK cells. Blue: isotype control; red: cytokine receptor antibody.

Supplemental Experimental Procedures

ELISA reagents used in this study

All ELISA antibodies, CCL2 (88-7391), IFN- γ (88-7314), GM-CSF (88-7334), IL-1 β (88-7013), IL-2 (88-7024), IL-4 (88-7044), IL-6 (88-7064), IL-10 (88-7104), IL-12 p40 (88-7120), TGF- β 1 (88-8350), TNF- α (88-7324), IL-17A (88-7371), were all from eBioscience.

FACS sort

6-8 week-old female mice were used in all sorting experiments. In general, 15-20 mice of the same genotype were used for FACS sorting in order to obtain sufficient stem and progenitor cells. Bone marrow cells were harvested from tibias and femurs and treated with red blood cell (RBC) lysis buffer (Biolegend) for 5 minutes on ice. After RBC lysis, bone marrow cells were stained with a cocktail of biotinylated lineage antibodies (CD11b, Gr1, CD19, B220, CD4, CD8, NK1.1, Ter119, and IL7R α , all 1:200 dilution) (eBioscience or Biolegend) and subjected to magnetic bead selection (Miltenyi) according to manufacturer's protocol to deplete lineage positive cells. After lineage depletion, cells were stained with fluorophore-conjugated antibodies (streptavidin 1:500 dilution, c-Kit 1:200 dilution, Sca1 1:200 dilution, CD150 1:100 dilution, CD48 1:100 dilution, Flt3 1:200, CD34 1:50) (eBioscience or Biolegend) for FACS sorting on a BD FACS Aria sorter at the Caltech FACS Core. 7-AAD was used to exclude dead cells during sorting.

Single cell cytokine chip analysis

We integrated upstream FACS purification techniques with the single cell barcode chip to study the functional proteomics from phenotypically defined single cells. The chips used in this study have >5000 microchambers of about 100 picoliters each in size to enable sensitive detection of proteins. Within each microchamber, a panel of 12 cytokines (TNF- α , GM-CSF, IL-6, IL-12p40, IFN- γ , IL-2, IL-4, IL-10, TGF- β 1, CCL-2, IL-17A and IL-1 β) can be simultaneously measured by a sandwich ELISA-like assay. The manufacture procedure of the chip has been described in detail in our previous study (Ma et al., 2011). Prior to each experiment, chips were first blocked with 3% BSA/PBS buffer, hybridized with an antibody-ssDNA conjugate cocktail and then washed 3 times with 3% BSA/PBS. Phenotypically defined cells were purified by FACS. LSK (Lineage⁻cKit⁺Sca1⁺), LT-HSCs (LSK CD150⁺CD48⁻ or LSK Flt3⁻CD34⁻), ST-HSCs (LSK Flt3⁻CD34⁺), MPPs (LSK Flt3^{int}CD34⁺) and LMPPs (LSK Flt3^{hi}CD34⁺) were purified from mouse bone marrows. After sorting, cells were re-analyzed on a FACS machine to ensure purity and viability immediately prior to loading onto a chip. Cells were suspended in culturing medium (RPMI plus sodium glutamate, non-essential amino acids and HEPES, 10% FBS and 50 μ M beta-mercaptoethanol). Appropriate stimuli, LPS (100ng/ml) or LPS (100ng/ml) plus Pam3CSK4 (1 μ g/ml), were added to the culturing medium. About 30,000 cells per chip were flown in through microfluidic device and cells would randomly settle into each of the >5000 microchambers. In general, based on the Poisson distribution, about 25% of the microchambers would have only one cell, while the rest would have zero cell, two cells, three cells and so forth. Cells were then cultured at 37 degree in 5% CO₂ cell incubator with medium alone, medium plus LPS, or medium plus LPS and Pam3CSK4 for 12 hours. At the end of stimulation, the chip was imaged using high resolution bright field microscope. Cell number in each chamber was counted and cell viability was assessed to exclude fragmented or non-light reflective cells blindly by trained personnel. Then cells were washed off. Secondary biotinylated detection antibodies and then streptavidin-cy3 were flown through to complete the immuno-sandwich assay. Finally, the slide was washed with 3% BSA/PBS, 50%/50% PBS/DI water and water in sequence. GenePix 4400A microarray scanner was used to scan slides and data were analyzed with GenePix Pro 7 software. Each single cell barcode chip analysis of a specific cell subset, genotype, and stimulating condition was performed at least two times.

Computational algorithm and statistical analysis

In Figure 3 and 5, F-tests were used to compare variances and then the appropriate two-sided student t-tests were applied. For all figures with error bars, they were graphed as mean \pm standard error of the mean (s.e.m.); for all heatmaps, scale bars were presented as mean \pm standard deviation (s.d.). * indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$.

For all single cell cytokine chip analysis, custom written software routines in R language were used to process, analyze and visualize the single cell functional assay results. Briefly, the algorithm converts raw fluorescence images into numerical fluorescence intensity values for each assayed protein within a given microchamber, matched with the number of cells. Number of cells within each microchamber was determined manually by microscopy. The fluorescence histograms were generated for 0, 1, 2 and 3-cell microchambers, so the average background signal levels from all 0-cell microchambers was used to set the gate to separate non-producing cells from cytokine-producing cells for 1, 2 and 3-cell microchamber (see our previous publication (Ma et al., 2011) for details). Any cells below the gate were non-producing cells and given a cytokine intensity value of 0 and any cells above the gate were producing cells and given the corresponding value (measured fluorescence intensity minus the background signal).

The data was used to generate one-dimensional and two-dimensional scatterplots. To look at all 12 proteins together by conducting clustering and principal component analysis, each protein was normalized by the average intensity (mean) and the standard deviation. For clustering analysis (e.g. Figure 1D), the data was analyzed by standard unsupervised two-way hierarchical clustering analysis to group similar proteins together and similar cells together. Then each cell group was re-plotted onto the 2D dimension represented by the top two principal components (PC1 and PC2). This two-dimension space explains the majority of information contained from the original 12-dimension cytokine space. The direction of the cytokines was identified by the projection of these cytokines onto the 2D space. The further outward the points lie in one direction from zero, the more strongly the cells are producers of this group of cytokines.

For Figure 2D and 2E, to look at properties of different cell types and cytokine intensity, 12-dimensional dataset comprising 12 different cytokine intensities from 6 different cell populations were reduced to two principal components PC1 and PC2. PC1 and PC2 were normalized to their ranges. Then the 6 cell populations were binned across the range of PC1 and PC2 to calculate their relative frequency. The relative frequency and the average intensity were normalized to show the comparative changes. This type of statistical analysis and graphical representations are routinely used to analyze large-scale multi-dimensional dataset from numerous cell subsets (Bendall et al., 2011).

Supplemental References

Bendall, S. C., Simonds, E. F., Qiu, P., Amir el, A. D., Krutzik, P. O., Finck, R., Bruggner, R. V., Melamed, R., Trejo, A., Ornatsky, O. I., *et al.* (2011). Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. *Science* 332, 687-696.

Ma, C., Fan, R., Ahmad, H., Shi, Q., Comin-Anduix, B., Chodon, T., Koya, R. C., Liu, C. C., Kwong, G. A., Radu, C. G., *et al.* (2011). A clinical microchip for evaluation of single immune cells reveals high functional heterogeneity in phenotypically similar T cells. *Nat Med* 17, 738-743.